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# Role of Superoxide Dismutase and Catalase as Determinants of Pathogenicity of *Nocardia asteroides*: Importance in Resistance to Microbicidal Activities of Human Polymorphonuclear Neutrophils

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**The roles of nocardial superoxide dismutase (SOD) and catalase in the resistance of *Nocardia asteroides* to the microbicidal properties of human polymorphonuclear leukocytes were determined in vitro. The neutrophils killed ca. 80% of the cells of the less virulent *N. asteroides* 10905 and ca. 50% of the log phase of the more virulent *N. asteroides* GUH-2 after 180 min of incubation. These phagocytes were not able to kill early-stationary-phase cells of strain GUH-2 that contained 10 times more intracytoplasmic catalase than log-phase cells of the same culture. However, the polymorphonuclear leukocytes were able to kill more than 50% of the cells of early-stationary-phase strain GUH-2 after treatment with purified antibody specific for surface-associated SOD. No killing was observed when the bacteria were treated with normal rabbit immunoglobulin G or with serum obtained from rabbits immunized against whole nocardial cells (containing little or no activity against SOD). These phagocytes killed more than 99% of *Listeria monocytogenes* used as a control. Chlorpromazine-treated polymorphonuclear leukocytes killed *L. monocytogenes* (70%) but they were not able to kill antibody-treated cells of *N. asteroides* GUH-2. Exogenously added SOD partially protected strain 10905, which lacked surface-associated enzyme, but it had no effect on the killing of strain GUH-2, which already possessed significant amounts of surface-bound SOD. In contrast, catalase added to the nocardiae provided almost complete protection to the log-phase cells of strain GUH-2, but strain 10905 was only partially protected. SOD combined with catalase had additive activity which completely protected the cells of strain 10905. A mutant of *N. asteroides* GUH-2 (SCII-C) is more virulent during the log phase than is the parental strain. This mutant contained at least 7 times more catalase at this stage of growth than did the parent. No other differences between these two strains were observed during the log phase. In sharp contrast to those of the parent, log-phase cells of this high-catalase mutant were not killed by polymorphonuclear phagocytes. These data indicate a role for both SOD and catalase in the resistance of *Nocardia* spp. to human neutrophils, and they represent at least two factors associated with virulence.**

Previous studies have shown that the relative virulence of *Nocardia asteroides* for mice is growth stage dependent (4). Furthermore, the resistance of nocardial cells at different phases of growth to the microbicidal activities of the phagocytes of the host is altered (2). Thus, log-phase cells of *N. asteroides* are significantly more virulent than stationary-phase cells of the same culture, but paradoxically they are more readily killed by activated macrophages and polymorphonuclear leukocytes (PMNs) than are stationary-phase cells (2, 4). Two of the mechanisms whereby virulent strains of *N. asteroides* survive within macrophages appear to be associated with the ability of these cells to inhibit phagosome-lysosome fusion and to alter the activities of lysosomal enzymes (7–9). Log-phase cells of *N. asteroides* GUH-2 inhibit fusion more effectively than do stationary-phase cells, although at the same time there appears to be an increased susceptibility to killing by these phagocytes (9). Both PMNs and activated macrophages possess oxygen-dependent and oxygen-independent microbicidal mechanisms. The oxygen-independent microbicidal activities of the phagocyte are probably associated with phagosome-lysosome fusion, whereas the oxygen-dependent mechanisms of killing probably do not rely upon fusion of lysosomes with the phagocytic vacuole (6). Therefore, the enhanced ability of phagocytes to kill log-phase cells of *N. asteroides*, even though there is increased inhibition of phagosome-lysosome fusion, may be related to the oxygen-dependent microbicidal activities of these phagocytes (6).

Virulent strains of *N. asteroides* in the early stationary phase of growth were not killed by PMNs, even though they induced an oxidative metabolic burst upon contact with these phagocytes (12). The virulent strain, *N. asteroides* GUH-2, was shown to secrete a unique superoxide dismutase (SOD) into the growth medium, and this SOD became surface associated. In contrast, no detectable catalase activity was found in the medium but remained localized within the cytoplasm. The less virulent strain, *N. asteroides* 10905, is killed by PMN more effectively than *N. asteroides* GUH-2 and has less SOD and catalase activity (5). These observations suggest a possible role for SOD and catalase in nocardial resistance to the killing mechanisms of PMNs and to their relative degrees of virulence. The studies presented below indicate the roles of both SOD and catalase in the growth-stage-dependent resistance of *N. asteroides* to the oxygen-dependent microbicidal activities of human PMNs.

## MATERIALS AND METHODS

**Microorganisms.** *N. asteroides* GUH-2 was isolated from a person with a fatal infection at Georgetown University, Washington, D.C., and *N. asteroides* 10905 was originally supplied by J. Rozanis, University of Western Ontario, London, Ontario, Canada. Mutants of *N. asteroides* GUH-2

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were obtained after UV irradiation and characterized as previously described (17). The strains of *N. asteroides* were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), as described previously. The specific growth curves were monitored, and single cell suspensions at specific stages of growth were prepared by removing clumps of bacteria onto loosely packed glass wool columns and then by differential centrifugation as previously described (10). The cells were pelleted and suspended in the appropriate reaction mixture, counted on a hemacytometer, and diluted to the desired cell concentration. *Listeria monocytogenes* was obtained from G. Filice, Stanford University, Stanford, Calif., and maintained as described above.

**Isolation of SOD.** Cells of *N. asteroides* GUH-2 were collected, washed, and broken open by using a $CO_2$-cooled Braun homogenizer (Allen-Bradley, Milwaukee, Wis.). The resultant cytoplasmic extracts were treated with ammonium sulfate, and the SOD was subsequently purified by using DEAE-cellulose chromatography as described previously (5). Quantitation, visualization, and determination of the specific activity and purity of each sample were done as previously described (5).

**Isolation of catalase.** Cytoplasmic extracts of *N. asteroides* GUH-2 were prepared as described above for the isolation of SOD and then extracted with ammonium sulfate in a stepwise manner to 30, 50, 65, and 95% saturation, followed by centrifugation at 30,000 × *g* to sediment the resulting precipitates. The catalase activities were tested in each fraction, and the fraction that contained the highest activity was purified further. The sample was dialyzed three times against 2 liters of TE buffer (0.01 M Tris-hydrochloride, 0.1 mM EDTA [pH 7.8]). The catalase was purified further by using batch DEAE-cellulose chromatography followed by gradient DEAE-cellulose chromatography. The fractions containing catalase were concentrated by using an Amicon concentrator with a ×M 100 membrane. The fractions were then purified further by Sepharose 4B chromatography and then concentrated, and the purity of the catalase was determined by both anionic and sodium dodecyl sulfate gel electrophoresis with disc gel and slab gel procedures as described previously (5). The specific activity of the catalase was determined by the methods described by Sigma Chemical Co. (Sigma technical bulletin no. 99, Feb. 1972).

**Preparation of antibody against purified SOD and catalase.** Antiserum was prepared by injecting rabbits at weekly intervals with three doses of either purified SOD or catalase in polyacrylamide adjuvant or Freund incomplete adjuvant. One week after the final injection, 50 ml of blood was removed and the serum was collected and fractionated with ammonium sulfate. The fraction at 40% saturation was collected, dissolved in water, dialzyed in 20 mM Tris-hydrochloride buffer (pH 7.5), absorbed onto a column of DE-52, and eluted with 0.0175 M phosphate buffer (pH 6.3). The fractions containing antibody were concentrated on a PM-10 Amicon membrane and stored at −80°C. Serum from unimmunized control rabbits was prepared in the same manner. The purity and titer of the antibody were determined by gel diffusion against cytoplasmic extracts and by radioimmunoassay (8). The nocardial SOD was incubated with dilutions of anti-SOD antibody, and the specific activity of the enzyme was determined. It was shown that the antinocardial SOD immunoglobulin G (IgG) fraction effectively inhibited SOD enzyme activity.

**Purity of enzymes and antibody.** By using combinations of polyacrylamide gel electrophoresis, spectrophotometric scans, protein determinations, immunodiffusion assays, radioimmunoassay, amino acid analysis, and immunofluorescence labeling as described previously (5), it was shown that the purity of the SOD and the catalase were >99 and >95%, respectively, and that the SOD samples contained no detectable catalase activity, whereas the purified catalase had no detectable SOD. When the purified immunoglobulin fraction obtained from the immunized rabbits was reacted against concentrated cytoplasmic extracts of *N. asteroides*GUH-2, it gave only one precipitin band each for SOD and catalase.

**Catalase and SOD activities of *N. asteroides* during the growth curve.** At specific stages of growth in brain heart infusion broth, the cells of *N. asteroides* GUH-2, GUH-2-SCII-AI, GUH-2-SCII-C, and 10905 were harvested and broken in a Braun homogenizer as described above, and the protein content of the cytoplasmic extract was determined by the standard Lowry assay, spectrophotometric assay at 260/280 nm, and Coomassie blue assay as described previously (5). The specific activities of SOD and catalase were quantitated as described above.

**Preparation of human PMNs and bactericidal assays.** Samples (ca. 200 ml) of heparinized blood (2 IU of Panheparin per ml of blood) were centrifuged at 700 × *g* for 15 min. The buffy coat was aseptically removed and diluted with an equal volume of sterile saline (0.85%). The diluted buffy coat was layered on lymphocyte separation medium (Bionetics, Kensington, Md.) and centrifuged at 700 × *g* at ambient temperature for 15 min. The bottom layer, consisting of erythrocytes and PMNs, was collected and washed in Hanks balanced salt solution, and the erythrocytes were lysed in EDTA-buffered ammonium chloride for 5 min at 5°C. The samples were washed three times in Hanks balanced salt solution, and the PMNs were counted by using a hemacytometer. Viability was determined by trypan blue exclusion. The PMNs were suspended in medium 199 containing 10% autologous human serum at a cell concentration of $10^7$ PMNs per ml. The neutrophil suspension was added to sterile 96-well microtest tissue culture plates (Becton Dickinson & Co., Oxnard, Calif.) at a concentration of $10^6$ PMNs per well (0.1 ml), and 0.1 ml of each reaction mixture containing ca. $10^6$ bacteria was also added. The plates were incubated at 37°C in a $CO_2$ incubator on a rocking platform to gently agitate the mixture to facilitate contact between the neutrophils and the bacteria. At specific times (0, 60, 90, and 180 min), the contents of each well (each sample in triplicate) were added to sterile distilled water blanks (1.8 ml) and incubated for 15 min. The distilled water-disrupted PMN preparations were diluted and plated in duplicate on tryptic soy agar plates. By using this procedure, it was determined that quantitative recovery of the bacteria was obtained, and the results were identical to those obtained by using a sonicator probe to lyse the PMN preparations (12).

**Role of surface-associated SOD in nocardial resistance to neutrophils.** The role of surface-associated SOD in nocardial resistance to the bactericidal effects of PMNs was determined by using antibody active against nocardial SOD. Single cell suspensions of *N. asteroides* GUH-2 ($2.0 \times 10^7$ CFU/ml) were incubated in either normal rabbit IgG or rabbit antinocardial SOD IgG (0.1 mg of protein per 0.1 ml) for 30 min at 37°C. Log-phase cells of *L. monocytogenes* were used as a control for the bactericidal capacity of the PMN preparation; ca. $3.4 \times 10^6$ PMNs were incubated with $2 \times 10^6$ CFU of either *N. asteroides* GUH-2 (48-h culture) or *L. monocytogenes* (18-h culture) as described above by using 96-well microtest tissue culture plates. All samples were done in triplicate and all dilutions were plated in duplicate. All experiments were done at least twice with the

same results. The wells included the following reaction mixtures: (i) *N. asteroides* plus normal IgG plus PMNs; (ii) *N. asteroides* plus normal IgG (no PMNs); (iii) *N. asteroides* plus anti-SOD IgG plus PMNs; (iv) *N. asteroides* plus 1:5 dilution of anti-SOD IgG plus PMNs; (v) *N. asteroides* plus anti-SOD IgG plus chlorpromazine-treated PMNs; (vi) *N. asteroides* plus normal IgG plus chlorpromazine-treated PMNs; (vii) *N. asteroides* plus anti-SOD IgG (no PMNs); (viii) *L. monocytogenes* plus normal IgG plus PMNs; (ix) *L. monocytogenes* plus normal IgG plus chlorpromazine-treated PMNs; (x) *L. monocytogenes* plus normal IgG (no PMNs). Samples were taken at zero time and 60, 120, and 180 min after adding the bacteria to the PMN suspension. The killing assays were performed as described above.

**Determination of the role of exogenously added SOD and catalase to nocardial resistance.** *N. asteroides* GUH-2 (16-h culture) and *N. asteroides* 10905 (48-h culture) were grown to the mid-log phase of growth in brain heart infusion broth as described above. The cells were harvested and washed, and single cell suspensions were prepared in medium 199 plus 10% plasma. Dilutions of either purified nocardial SOD (specific activity, 1,330 U/mg), catalase (specific activity, 8,990 U/mg), or SOD and catalase were added to the bacterial suspensions and incubated for 30 min. Samples (ca. $10^6$ CFU per well) of nocardiae in the appropriate enzyme mixtures were added to sterile 96-well microtest tissue culture plates (0.1 ml per well) with $10^6$ PMNs. At 0, 90, and 180 min, the samples from each well were diluted in distilled water, and plate counts were done as described above. All samples were done in triplicate, plated in duplicate, and repeated at least twice. *L. monocytogenes* was used as a control for PMN function. The microtiter wells included the following reaction mixtures: (i) *N. asteroides* plus catalase plus PMNs; (ii) *N. asteroides* plus catalase plus SOD plus PMNs; (iii) *N. asteroides* plus SOD plus PMNs; (iv) *N. asteroides* plus catalase; (v) *N. asteroides* plus SOD; (vi) *N. asteroides* plus catalase plus SOD; (vii) *N. asteroides* plus media alone; (viii) *N. asteroides* plus catalase plus chlorpromazine-treated PMNs; (ix) *N. asteroides* plus SOD plus chlorpromazine-treated PMNs; (x) *N. asteroides* plus catalase plus SOD plus chlorpromazine-treated PMNS; (xi) *N. asteroides* plus untreated PMNs; (xii) *N. asteroides* plus chlorpromazine-treated PMNs; (xiii) *L. monocytogenes* plus PMNs; (xiv) *L. monocytogenes* plus chlorpromazine-treated PMNS; (xv) *L. monocytogenes* plus media alone.

**High-catalase mutant of *N. asteroides* GUH-2.** Mutants of *N. asteroides* GUH-2 were induced by exposure to UV irradiation. These mutants were selected and characterized as described by Vistica and Beaman (17). Mutant strain SCII-C was found to be significantly more virulent than the parental strain (GUH-2-SCII-P) during the log phase of growth, whereas mutant strain SCII-AI was consistently less virulent than strain GUH-2-SCII-P (17). It was found that the chemical composition of the cell envelope of SCII-AI differed substantially from that of the parent, whereas the cell wall of SCII-C was almost indistinguishable from that of the parent. Thus, the decreased virulence of SCII-AI appears to reside within the chemical makeup of the cell wall. In contrast, it was observed that strain SCII-C during the log phase of growth had considerably higher levels of catalase than did the parental strain at the same stage of growth. The surface-associated SOD levels in strain SCII-C appeared to remain the same as those in strain GUH-2-SCII-P. The catalase levels and SOD composition of the less virulent mutant SCII-AI appeared to be similar to those of strain GUH-2-SCII-P. To study further the relationship between the levels of intracellular catalase, virulence, and resistance to the microbicidal activities of PMNs, killing assays were performed with the mutants at both log and early stationary phases of growth as described above.

## RESULTS

Previous studies showed that SOD was secreted into the medium during growth of the virulent strain *N. asteroides* GUH-2. Furthermore, this SOD became cell surface associated, and the amount of total SOD detected per CFU was growth stage dependent. Thus, cells in the early log phase produced more SOD per CFU than did cells in the stationary phase (5). The data presented in Fig. 1 show that this strain of *N. asteroides* produced large amounts of intracytoplasmic catalase with activity that was also shown to be growth stage dependent. Maximal levels of catalase were found during the early stationary phase (1,482 U/mg of protein), whereas significantly decreased levels of activity (ca. 10-fold) per CFU were observed during the log phase (113 U/mg of protein). Thus, with *N. asteroides* GUH-2-SCII-P, catalase levels were increased at the time that SOD levels decreased. These growth stage-dependent changes in SOD and catalase were not observed with the less virulent *N. asteroides* 10905 (Table 1) (5). Furthermore, the more virulent mutant GUH-2-SCII-C possessed high levels of catalase throughout its growth cycle (Table 1).

The role of surface-associated SOD in the resistance of *N. asteroides* to the microbicidal activities of PMNs was studied by using antibody prepared against purified nocardial SOD (Fig. 2). Human PMNs were able to kill more than 99% of the cells of *L. monocytogenes* (used as a control on PMN function) in 3 h, whereas in contrast, they were not able to kill early-stationary-phase cells of *N. asteroides* GUH-2 (Fig. 2). Antinocardial SOD antibody (rabbit IgG) did not

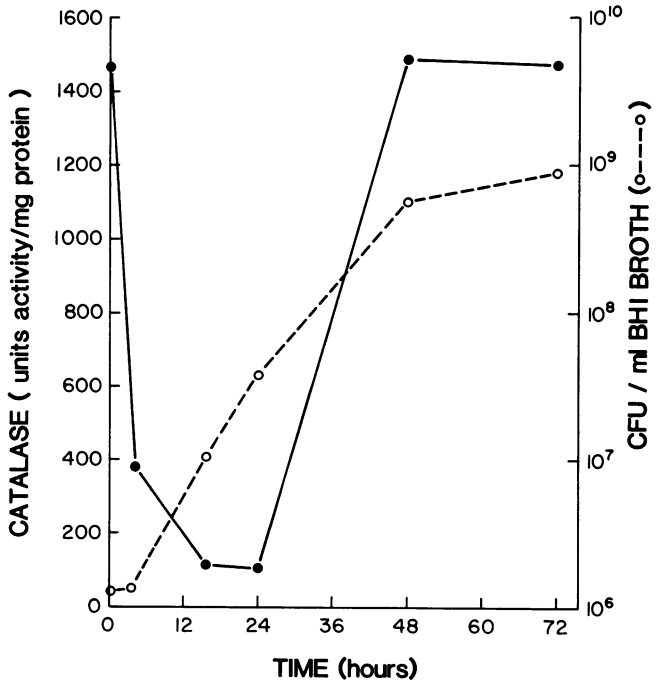


FIG. 1. Relationship of the rate of growth of *N. asteroides* GUH-2 and the intracytoplasmic activity of catalase. Symbols: ○, CFU/ml of brain heart infusion broth; ●, catalase, U/mg of protein.

TABLE 1. Catalase activity in the cytoplasm of *N. asteroides* at different stages of growth

| Strain (stage of growth) | Sp act of catalase (U/mg of protein) |
|---|---|
| *N. asteroides* GUH-2-SCII-P (log) | 113 |
| *N. asteroides* GUH-2-SCII-P (early stat) | 1,482 |
| *N. asteroides* GUH-2-SCII-C (log) | 923 |
| *N. asteroides* GUH-2-SCII-C (early stat) | 1,170 |
| *N. asteroides* 10905 (log) | 220 |
| *N. asteroides* 10905 (early stat) | 350 |
| *L. monocytogenes* (late log-early stat) | 120 |
| *E. coli* K-12 (late log-early stat) | 22 |

have any detectable effect on the ability of PMNs to kill *L. monocytogenes*; however, ca. 50% of the cells of *N. asteroides* GUH-2 treated with anti-SOD IgG were killed within 3 h of incubation ($P = <0.001$) (Fig. 2). A fivefold dilution of this antibody before preincubation with the nocardial cells abrogated the effect. Normal rabbit IgG as well as hyperimmune sera prepared against whole cells of *N. asteroides* GUH-2 (containing little or no detectable antibody specific against SOD) had no effect on PMN killing of *N. asteroides*. Thus, the antibody directed against nocardial SOD was specific in its ability to decrease the resistance of *N. asteroides* GUH-2 to the microbicidal activities of PMNs (Fig. 2). It was shown that the neutrophils killed the antibody-treated nocardiae by way of oxidative metabolism, since this killing was eliminated by chlorpromazine, which inhibits the oxidative metabolic burst of PMNs. (It should be noted that chlorpromazine may affect other functional capacities of PMNs.) The chlorpromazine reduced the ability of the PMNs to kill *L. monocytogenes* at ca. 70% in 3 h. In contrast to their effect on *Nocardia* strains, these results indicate that PMNs kill *Listeria* strains by both oxidative and nonoxidative mechanisms (Fig. 2).

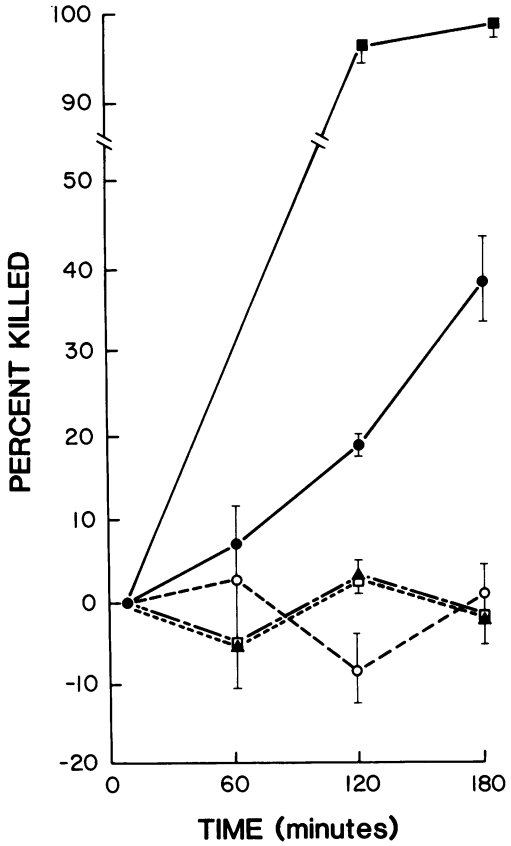


FIG. 2. Effect of anti-nocardial SOD antibody on the ability of human PMNs to kill cells of *N. asteroides* GUH-2 (early stationary phase). Symbols: ●, *N. asteroides* plus antibody plus PMNs; □, *N. asteroides* plus PMNs (no antibody); ▲, *N. asteroides* plus chlorpromazine-treated PMNs (no antibody); ○, *N. asteroides* plus antibody plus chlorpromazine-treated PMNs; ■, *L. monocytogenes* control plus PMNs. The IgG fraction was used at a concentration of 10 mg/ml; 50 μl/$10^6$ CFU. Bars represent standard deviation.

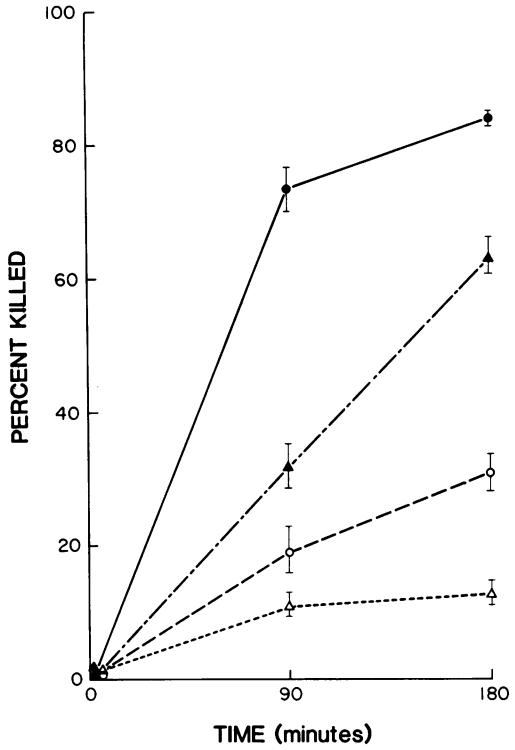


FIG. 3. Effect of exogenously added nocardial SOD and catalase on the ability of human PMNs to kill cells of the less virulent *N. asteroides* 10905. Symbols: ●, *N. asteroides* plus PMNs (no enzyme); ▲, *N. asteroides* plus SOD plus PMNs; ○, *N. asteroides* plus catalase plus PMNs; △, *N. asteroides* plus catalase plus SOD plus PMNs. Specific activity of SOD added = 1,330 U/mg; specific activity of catalase added = 8,990 U/mg. Bars represent standard deviation.

It was observed that the less virulent strain, *N. asteroides* 10905, does not have detectable surface-associated SOD, does not secrete SOD into the growth medium, and has less intracellular SOD than the virulent *N. asteroides* GUH-2 (5). Therefore, 48-h cultures of *N. asteroides* 10905 were incubated with PMNs and the microbicidal activity was determined. Human neutrophils killed ca. 80% of the nocardiae in 3 h, whereas chlorpromazine-treated phagocytes killed ca. 15% of the bacteria ($P = <0.001$) (Fig. 3). Exogenously added purified nocardial SOD reduced the ability of the PMNs to kill *N. asteroides* 10905 to ca. 60% in 3 h. Since the virulent strain GUH-2 has significantly higher levels of catalase than cells of strain 10905 (Table 1), nocardial catalase was purified from strain GUH-2 and exogenously added to strain 10905. Catalase alone reduced the ability of PMNs to kill strain 10905 better than exogenously added SOD did. The dilution of either SOD or catalase resulted in

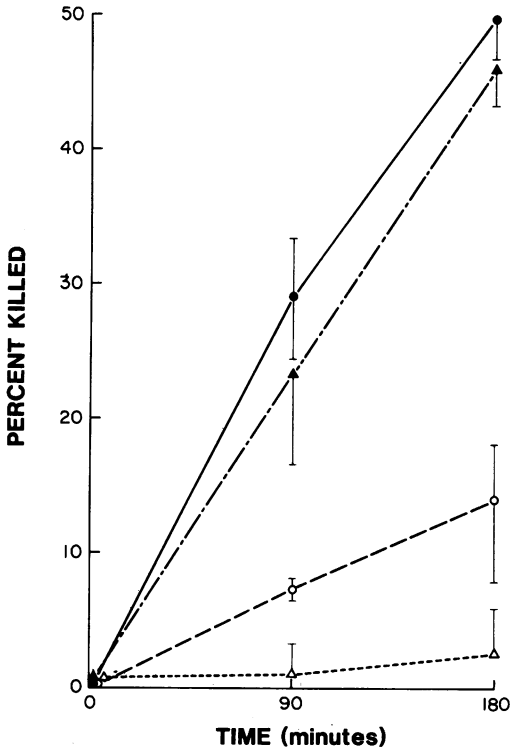


FIG. 4. Effect of exogenously added nocardial SOD and catalase on the ability of human PMNs to kill cells of the virulent *N. asteroides* during the log phase of growth. Symbols: ●, *N. asteroides* GUH-2 plus PMNs (no enzyme); ▲, *N. asteroides* plus SOD plus PMNs; ○, *N. asteroides* plus catalase plus PMNs; △, *N. asteroides* plus catalase plus SOD plus PMNs. For specific activity of SOD and catalase, see the legend to Fig. 3. Bars represent standard deviation.

corresponding changes in the susceptibility of *N. asteroides* 10905 to the PMN suspension. However, either SOD or catalase added alone to the nocardiae, even in high concentrations, was unable to protect the nocardiae completely. When SOD and catalase were combined simultaneously to the cultures, the killing was reduced to ca. 15% ($P < 0.001$). This was the same degree of killing of *N. asteroides* 10905 observed in chlorpromazine-treated PMN preparations; therefore, these phagocytes were able to kill ca. 15% of the nocardial cells nonoxidatively (Fig. 3). Diluting the combinations of SOD and catalase respectively indicated that the effects of these two enzymes may be additive in their ability to protect the cells of nocardiae from the microbicidal activities of PMNs (data not shown).

It has been noted previously that log-phase cells of *N. asteroides* GUH-2 were significantly more virulent than stationary-phase cells of the same culture, and yet these cells were also more readily killed by macrophages (2, 4). Quantitation of SOD activity demonstrated that log-phase cells had more SOD than did stationary-phase cells (5). In contrast, it was observed that log-phase cells of strain GUH-2 contained over 10 times less catalase activity than early-stationary-phase cells of the same culture (Table 1). Early-stationary-phase cells of *N. asteroides* GUH-2 were completely refractory to the microbicidal activities of PMNs. On the other hand, it was found that ca. 50% of the log-phase cells of *N. asteroides* GUH-2 were killed in 3 h by these phagocytes ($P < 0.001$) (Fig. 4). Exogenously added nocardial SOD had little or no effect on the resistance of log-phase cells of GUH-2 to the PMNs. These results are not surprising, since the log-phase cells were already producing maximal amounts of SOD that became surface associated (5). Exogenously added nocardial catalase reduced the ability of the PMNs to kill log-phase GUH-2 cells (ca. 12% killed in 3 h), and SOD plus catalase eliminated the susceptibility of log-phase GUH-2 cells to the microbicidal activities of the PMNs ($P < 0.001$) (Fig. 4). Chlorpromazine-treated PMNs were not able to kill the log phase of strain GUH-2.

Mutants of *N. asteroides* GUH-2 were induced by UV irradiation, and their relative virulence was determined during their growth cycle (17). One mutant, GUH-2-SCII-C, was found to be at least twice as virulent during the log phase of growth than the parental strain GUH-2 (17). Studies of the cell envelope, cellular composition, and physiological properties of this mutant failed to show significant differences between it and the parent, except that during the log phase GUH-2-SCII-C had 8 to 10 times higher levels of catalase (923 U/mg of protein) than GUH-2-SCII-P at log phase (113 U/mg of protein) (Table 1). It was shown that, unlike the log phase of GUH-2-SCII-P, the log phase of the mutant SCII-C was not killed (0%) ($P < 0.001$) by PMNs (Fig. 5).

## DISCUSSION

The data presented above indicate that there is a relationship among the production of a surface-associated and secreted SOD, high levels of catalase, phase of growth,

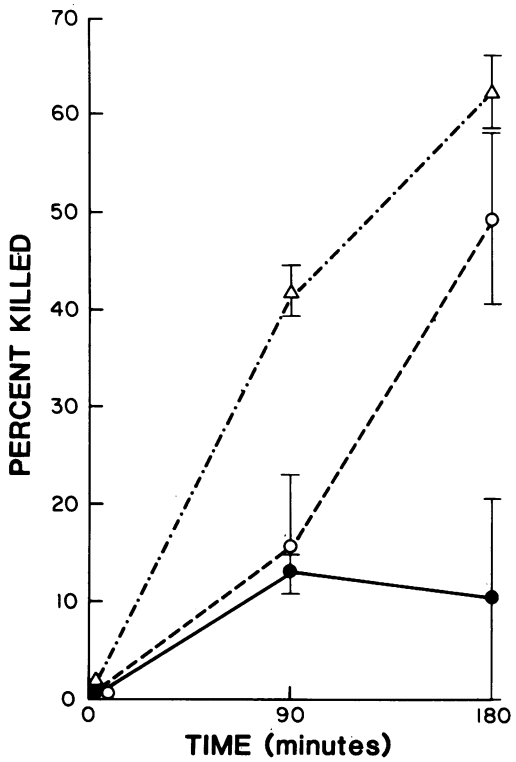


FIG. 5. Ability of human PMN neutrophils to kill the high-catalase mutant *N. asteroides* GUH-2-SCII-C during the log phase of growth. Symbols: △, *N. asteroides* 10905 plus PMNs (control); ○, *N. asteroides* GUH-2-SCII-A1 (log phase) plus PMNs (GUH-2-SCII-P at log phase gave the same results); ●, *N. asteroides* GUH-2-SCII-C (log phase) plus PMNs. The levels of endogenous catalase are shown in Table 1. Bars represent standard deviation.

virulence, and resistance of *N. asteroides* to the microbicidal activities of human PMNs. Thus, the less virulent strain, *N. asteroides* 10905, is more susceptible to the oxidative killing mechanisms of PMNs than is the more virulent *N. asteroides* GUH-2. Furthermore, exogenously added SOD and catalase purified from strain GUH-2 completely protect strain 10905 from the lethal effects of PMNs. Catalase and SOD act together in an additive way to protect the nocardiae. Specific antibody active against the surface-associated SOD enhances the susceptibility of *N. asteroides* GUH-2 to the lethal effects of the PMNs. Nocardial cells that have high levels of intracytoplasmic catalase are significantly more resistant than the cells from the same culture that contain 10-fold less catalase. Moreover, mutants of *N. asteroides* that produce more catalase at the log phase of growth are both more virulent for mice and more resistant to the microbicidal activities of PMNs.

Recently, Filice showed that *N. asteroides* GUH-2 is more resistant in vitro to the lethal effects of $H_2O_2$ or to a combination of $H_2O_2$ lactoperoxidase and iodide than is *Staphylococcus aureus* (11). It was reported that *N. asteroides* has significantly more catalase than does *S. aureus* (11). Similarly, we showed that cells of *N. asteroides* that were resistant to PMNs contained 10 times more catalase than cells of *L. monocytogenes* that were readily killed (>99% in 3 h) by these phagocytes. Furthermore, it was found that *Escherichia coli* K-12, a nonpathogen, contained between 5 and 67 times less catalase than did *N. asteroides*, depending upon the stage of growth.

Other studies have also suggested a relationship between SOD and the levels of intracytoplasmic catalase and the virulence of various pathogens. Several isolates of *Mycobacterium tuberculosis* have been analyzed, and the more virulent strains usually have more catalase than do the less virulent strains (13). Furthermore, virulent strains of *M. tuberculosis* were found to secrete SOD into the growth medium, whereas less virulent species of *Mycobacterium* did not (15). On the other hand, Mandell was unable to demonstrate a correlation between cytoplasmic SOD and the virulence of *S. aureus*, but there was a relationship between catalase and staphylococcal virulence (16). Thus, strains of *Staphylococcus* with high levels of catalase were virulent, whereas strains with low catalase activity were avirulent (16).

There is frequently a correlation between the resistance of a pathogen to the microbicidal effects of PMNs and its virulence. Mandell found that virulent strains of *Staphylococcus* with high levels of catalase were more resistant to oxidative killing mechanisms than the less virulent low-catalase strains (16). In addition, Mandell reported that catalase added exogenously to cells of *S. aureus* protected them from the microbicidal activities of PMNs; however, the addition of exogenous SOD had little effect on staphylococcal resistance to leukocytes (16). In contrast, Johnston et al. (14) showed that either SOD or catalase added exogenously protected cells of *S. aureus*, *Streptococcus viridans*, and *E. coli* from the lethal effects of PMNs. Studies in which both SOD and catalase were added simultaneously to microorganisms have not been reported.

The seemingly contradictory observations that log-phase cells are more readily killed by PMNs but are more virulent than stationary-phase cells of the same culture underscore both the complexity and multiplicity of the pathogenic mechanisms of *N. asteroides*. There are substantial growth stage-dependent alterations in the composition of the nocardial cell envelope during its growth cycle (1, 3). These structural and chemical shifts result in changes in host-parasite interactions (2, 4, 9). Thus, in macrophage preparations, log-phase cells of *N. asteroides* GUH-2 are less easily phagocytized and more toxic, grow more rapidly, inhibit phagosome-lysosome fusion more effectively, and have greater tropism for vital organs such as the brain when injected into mice than do stationary-phase cells of the same culture (2, 4, 9, 17). The phagocyte-induced killing of log-phase cells probably occurs during the early stages of phagocytosis by oxidative mechanisms in both macrophages and PMNs. The dramatic protection of nocardial cells provided by exogenously added catalase and SOD suggest that some of the nocardiae are being killed extracellularly by the products of oxidative metabolism released by the PMNs. In this regard, it should be noted that macrophages are quite distinct from PMNs in their interactions with nocardiae. Therefore, even though log-phase cells are more susceptible to the oxygen-dependent microbicidal activity of PMNs, they are also more resistant to the oxygen-independent killing mechanisms. As a result, *N. asteroides* and its mutants, at different stages of growth, may be used to study independently these two microbicidal components of phagocytes.

It is clear that the determinants of pathogenesis of *Nocardia* strains are complex and that no single cellular attribute can be defined as the mechanism of virulence. However, high levels of intracytoplasmic catalase combined with surface-associated and secreted SOD appear to be the primary mechanisms whereby *N. asteroides* avoids the oxidative killing mechanisms of PMNs; thus, catalase and SOD represent two important virulence factors for *Nocardia* strains.

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